Targeted elimination of leukemia stem cells; a new therapeutic approach in hemato-oncology

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ABSTRACT

Despite recent advances, treatment of leukemia is often not curative. New insights indicate that this may be attributable to a small population of therapy-resistant malignant cells with self-renewal capacity and the ability to generate large numbers of more differentiated leukemia cells. These leukemia-initiating cells are commonly referred to as Leukemia Stem Cells (LSCs). LSCs are regarded as the root of leukemia origin and leukemia recurrence after seemingly successful therapy. Not surprisingly therefore, contemporary leukemia research has focused on ways to specifically eliminate LSCs, leading to the identification of several promising anti-LSC strategies. Firstly, LSCs may be eliminated by antibody- or ligand-based cell surface delivery of therapeutics such as naked antibodies, immunotoxins, and immunocytokines. This approach exploits LSC-associated surface antigens, such as CD33, CD44, CD96, CD123 and CLL-1 for LSC-selective therapy and aims to spare normal hematopoietic stem cells. A second strategy aims to disrupt the interactions between LSCs and their highly specialized niche. These interactions appear to be pivotal for maintenance of the stem cell-like characteristics of LSCs. A third strategy centers on the selective modulation of aberrantly activated signaling pathways central to LSC biology. A fourth strategy, dubbed 'epigenetic reprogramming', aims to selectively reverse epigenetic alterations that are implicated in ontogeny and maintenance of LSCs. In this review, we will discuss the rationale for these LSCs-targeted strategies and highlight recent advances that may ultimately help pave the way towards selective LSCs-elimination.

INTRODUCTION

To a varying degree most leukemia types initially respond well to therapy with partial or even complete remissions. Unfortunately, after a period of minimal residual disease (MRD) many patients succumb to refractory relapses of the disease. Recent insight indicates that the development of these relapses may be due to the selective continued survival of a small, but distinct, population of therapy-resistant tumor-initiating cells, commonly referred to as Leukemia Stem Cells (LSCs). LSCs are thought to originate either from normal hematopoietic stem cells (HSCs) or from more differentiated progenitor cells that have acquired malignant features. In the latter case, the progenitor cells have de-differentiated and re-acquired stem cell-like characteristics via as yet undefined pathways [1;2]. Both LSCs and HSCs possess self-renewal capacity but are relative quiescent compared to more mature progenitors cells (see Fig. 1). However, LSCs typically have a much stronger capacity for cellular expansion than normal HSCs, probably due to an increase in symmetric self-renewal activity of LSCs [3].

LSCs were first identified in Acute Myeloid Leukemia (AML) by John Dick and colleagues [3;4] and have by now been found in all major forms of leukemia [3-6]. LSCs are experimentally defined as cells capable of serial engraftment in immunocompromised mice (see Fig. 1). Importantly, mice engrafted with these LSCs develop leukemia which resembles the patient's original disease [7]. During this last decade substantial progress in the field of LSC research has led to the identification of many of the hallmark

characteristics of LSCs. Therapy-resistance is typical for LSCs which is thought to arise from (1) their relative quiescent cellular activity [8;9], (2) their array of self-protecting mechanisms [10], and (3) the highly protective microenvironment in which the LSCs resides [11].

Since curative treatment of leukemia will most likely require the elimination of LSCs, new strategies need to be designed that overcome the above-described features. Importantly, in this design care should be taken to spare normal HSCs. In this respect, strategies that target a distinct Achilles' heel of the LSC biology appear to be particularly promising. Here, we review the rationale of different LSCs-specific strategies and highlight recent advances that may ultimately lead to selective LSCs-elimination.

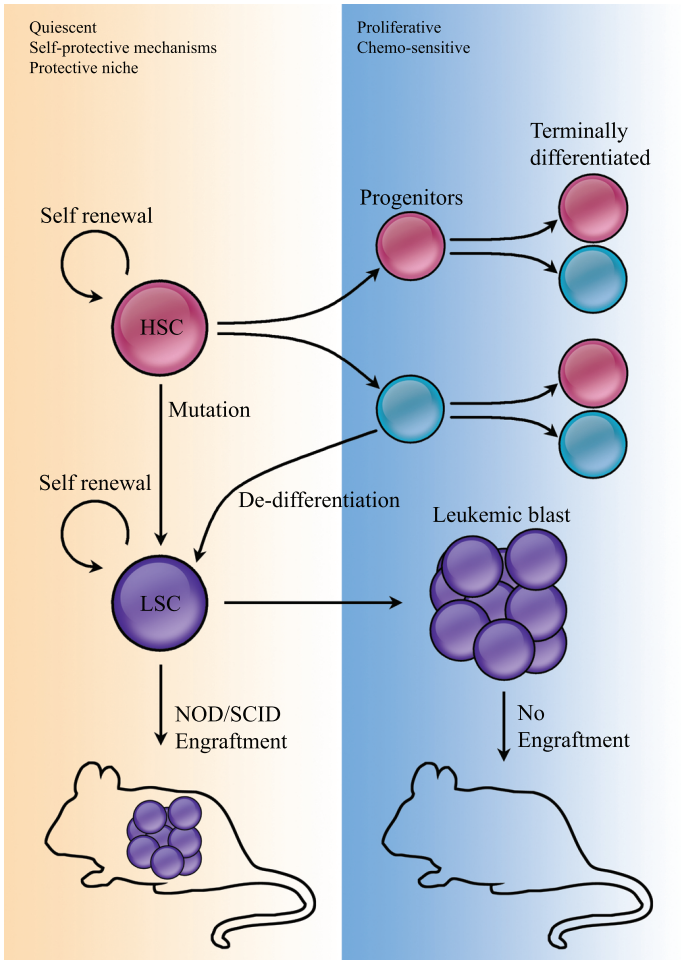

Figure 1. Hypotheses for the ontogeny of leukemia stem cells. *LSCs can arise by somatic mutations in normal hematopoietic stem cells (HSC) or more mature progenitor cells can become malignant, de-differentiate and re-acquire stem cell-like characteristics. Currently, LSCs (typically $CD34^{+}CD38^{-}$) are defined by the ability to engraft in immunocompromised mice and initiate leukemia resembling the original patient's disease.*

LSC CELL SURFACE TARGETING APPROACHES

Cell surface antigens expressed on LSCs may be exploited for antibody- or ligand-based therapeutic approaches. The ideal target antigen is exclusively and abundantly expressed on LSCs, thereby maximizing the therapeutic effect and minimizing off-target effects. Unfortunately, such an antigen has not been uncovered. However, a series of surrogate cell surface markers have been identified which have favorable LSCs-restricted expression profiles. These surrogate markers can be targeted with naked antibodies, which typically eliminate targeted cells via Antibody Dependent Cellular Cytotoxicity (ADCC) or Complement Dependent Cytotoxicity (CDC). However, some antibodies can directly interfere with the signaling activity by binding to receptor molecules and sensitize targeted cells to apoptosis induction [12] or directly induce apoptosis as reported for the anti-CD20 monoclonal antibody (mAb) Rituximab [13]. The tumoricidal potential of antibodies can be further enhanced by conjugating them to cytotoxic drugs or radionuclides. Alternatively, cell surface molecules may also be targeted using recombinant forms of their corresponding natural ligand. Ligand-based approaches have been used to deliver cytotoxic drugs to the target cell and to trigger apoptosis-inducing receptors. However, an obvious potential limitation to antibody-based and ligand-based therapy is the selective loss of the target antigen during therapy or the presence of target antigen-negative leukemia cells at the start of therapy. In this respect, fluctuations in the expression of cell surface markers on HSCs have been reported [14]. Therefore, the rational design of combinatorial antibody-based or ligand-based strategies that target two or more different LSC antigens simultaneously may prove the best way to prevent escape form therapy. Below, a selection of the most prominent cell surface molecules on LSCs and approaches for targeting these molecules are briefly reviewed (summarized in Fig. 2).

LSC Target antigens

CD33 belongs to immunoglobulin (Ig) superfamily and is a member of the sialoadhesin family of cellular interaction molecules [15;16]. The intracellular domain of CD33 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain which may function as a negative regulator of cellular differentiation [17]. CD33 expression is largely restricted to the myeloid lineage, however some expression is detected on certain peripheral blood lymphocytes. In leukemia, CD33 is expressed on blasts in the majority of AML patients. Importantly, it has been noted that CD33 is also expressed on AML-LSCs, but not on normal HSCs [18;19].

CD123 (IL-3Rα) is the alpha subunit that hetero-dimerizes with the IL3β chain to form the interleukin-3 receptor. CD123 is constitutively expressed on hematopoietic progenitors cells (HPCs), on which it is involved in proliferation and differentiation. Furthermore, CD123 is expressed on LSCs in AML [20], Chronic Myeloid Leukemia (CML), Myelodysplastic Syndrome (MDS) and Systemic Mastocytosis [18], but not on HSCs [21]. This expression profile indicates that CD123 might be a suitable target for the selective elimination of LSCs in various hematological malignancies.

C-type Lectin-Like Molecule-1 (CLL-1) is a heavily N-glycosylated transmembraneprotein with an intracellular ITIM domain [22]. CLL-1 is exclusively expressed on cells of the hematopoietic lineage, including dendritic cells, granulocytes and monocytes [23]. In leukemia, CLL-1 expression is detected on 92% of all AML cases [23] on which a high expression at diagnosis correlates with an early relapse [24]. Importantly, CLL-1 is also expressed on AML-LSCs, but not on HSCs [24;25]. In line with these findings, it was demonstrated that sorted $CD34^+/CLL\text{-}1^+$ AML cells are able to engraft and generate $CLL\text{-}1^+$ blasts after transplantation in Non-Obese Diabetic/ Severe Combined ImmunoDeficient (NOD/SCID) mice [24].

CD96 is a transmembrane protein belonging to the Ig superfamily. The extracellular domain of CD96 has three Ig-like regions and a stalk-like region which can be heavily O-glycosylated. The only ligand for CD96 identified thus far is CD155, also known as polio virus receptor [26]. On normal cells CD96 protein expression appears to be restricted to T and NK cells, while its expression was also detected on T cell -Acute Lymphoid Leukemia (ALL) cells and AML cells [27;28]. Recently, Hosen and colleagues identified that compared with normal HSCs in AML-LSCs CD96 mRNA levels are at least 200-fold increased [28]. These mRNA expression data suggests that CD96 protein may be selectively over-expressed on AML-LSCs. In line with this finding, they demonstrated that $CD96^+$ AML cells have a significantly higher engraftment potential then $CD96^-$ AML cells [28]. Therefore, the development of antibody-based or CD96 ligand-based therapeutics may have potential for the targeted elimination of $CD96^+$ AML-LSCs.

Targeting LSCs with naked antibodies

Indications for the efficacy of targeting $CD123^+$ AML-LSCs with naked antibodies is demonstrated by a preclinical study using the CD123 neutralizing antibody, mAb7G3 [29]. Treatment with mAb7G3 inhibited engraftment and homing of human $CD123^+$ AML-LSCs when injected in NOD/SCID mice. This effect of mAb7G3 was restricted to AML cells, since there was only a minor effect towards normal bone marrow (BM) cells. Furthermore, mAb7G3 treatment of NOD/SCID mice with pre-established AML reduced both the disease burden and the ability to transplant AML in secondary recipient mice (Lock R, et al. ASH Annual Meeting Abstracts 2007;110:161).

Recently, *in vitro* treatment with naked anti-CLL-1 mAbs in the presence of serum resulted in efficient Complement Dependent Cytotoxicity (CDC) towards various $CLL\text{-}1^+$ AML cell lines. In line with these findings, *ex vivo* treatment of primary AML blasts with naked anti-CLL1 mAbs resulted in CDC in all samples tested. Furthermore, in a xenograft model one particular anti-CLL-1 mAb reduced the growth of established tumors by 38% (Korver W, et al. ASH Annual Meeting Abstracts 2008;112:4003). Together, these data indicate that targeting both CD123 and CLL-1 by naked antibodies might be a promising approach for the selective elimination of LSCs.

Targeting LSCs with immunoconjugates

The expression of CD33 in AML has resulted in the development of several CD33-targeted experimental therapeutics [30;31] and the clinically applied anti-AML immunotoxin Gemtuzumab Ozogamicin (GO). GO consists of an anti-CD33 mAb that is chemically

coupled to a derivative of the calicheamicin-toxin. After CD33-selective binding, GO internalizes and ends up in the acidic milieu of lysosomes where the calicheamicin moiety is hydrolytically released. Subsequently, calicheamicin translocates to the nucleus and intercalates with the DNA, causing site-specific double-strand breaks resulting in apoptotic cell death [32]. In the United States, GO was approved by the FDA for use in patients over the age of 60 with relapsed AML who are not considered candidates for standard chemotherapy [33]. In this subset of patients, GO treatment resulted in a 26% remission rate, albeit with sometimes considerable toxicity [34]. Recently, we have demonstrated that the activity of GO is strongly enhanced when treatment is combined with the epigenetic modifying drug valproic acid (VPA), a histone deacetylase inhibitor (HDACi). The co-treatment of AML cells with GO and VPA facilitated the intercalation of calicheamicin with the DNA, followed by synergistic apoptosis induction [35].
It has been reported that *in vitro* treatment of AML-LSCs with GO results in a median decrease in cellular viability with 46%, while normal HSCs were largely unaffected (Jawad M et al. ASH Annual Meeting Abstracts 2007;110:650). Therefore, it would be interesting to assess whether the anti-LSCs activity of GO can be further enhanced when treatment is combined with VPA.
One potential resistance mechanism of AML-LSCs towards treatment with GO involves the increased expression and/or activity of drug efflux pumps. In this respect, it appears that P-glycoprotein (Pgp) expression confers resistance to GO and as such is associated with a worse clinical response [36]. Therefore, combination treatment of GO and Pgp inhibitors may enhance the therapeutic effect of GO towards Pgp-expressing LSCs.
Alternatively, efflux pump-related resistance in AML-LSCs may be overcome using an anti-CD33 antibody conjugated to cytolytic radionuclides like ^{111}In. In vitro experiments with an ^{111}In-conjugated anti-CD33 mAb resulted in potent apoptosis in both chemo-resistant CD33$^+$, HL-60 AML cells and in primary AML cells. Indeed, efflux pump-related resistance was overcome by ^{111}In since these AML cells expressed several drug efflux pumps including Pgp, Breast Cancer Resistance Protein 1 and Multidrug Resistance Protein 1 [37].

Currently, the efficacy of various CD123-targeted immunoconjugates is under investigation. In one particular approach CD123 is targeted by a fusion protein comprising a CD123-specific single chain fragment of the variable regions (scFv) antibody fragment that is genetically fused to a fragment of pseudomonas exotoxin A (PE38). This fusion protein, designated 26292(Fv)-PE38-KDEL, potently induced apoptosis in a variety of CD123$^+$ leukemia cell lines [38]. It remains to be investigated whether 26292(Fv)-PE38-KDEL can also eliminate CD123$^+$ LSCs.

Targeting LSCs with ligand-based approaches
The cytokine IL3 has been used in fusion protein format to selectively deliver diphtheria toxin (DT) to CD123$^+$ AML cells [39]. This IL3-DT fusion protein also selectively eliminated primitive AML progenitors, while normal progenitors where not affected [40]. Importantly, the IL3-DT fusion protein inhibited engraftment of human CD123$^+$ AML-LSC cells when injected in NOD/SCID mice [40;41]. Recently, it was demonstrated that the pro-apoptotic activity of IL3-DT is enhanced when treatment is combined with Tumor necrosis factor

Related Apoptosis Inducing Ligand (TRAIL) [42]. Currently, a phase I clinical trial with this IL3-DT fusion protein for a subset of AML and MDS patients is initiated [41].

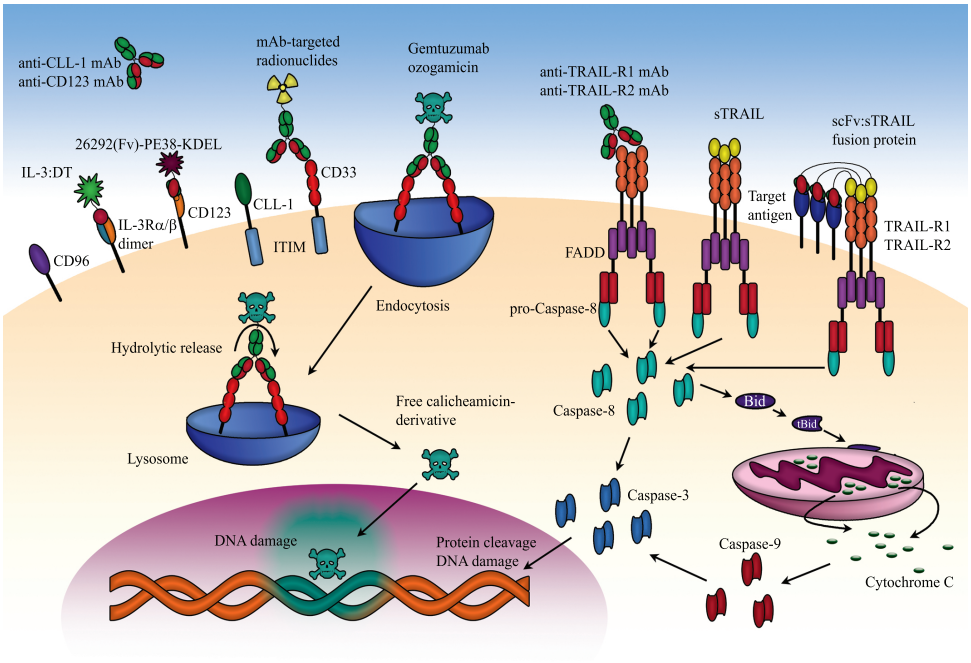

Figure 2. Antibody- and ligand-based approaches for the elimination of LSCs. *The cell surface molecules CD96, CD123 (IL3Rα), CLL-1, CD33 and TRAIL-R1/R2 may be exploited for the selective elimination of LSCs. CD123 targeted strategies include mAb7G3, a fusion protein comprising an CD123-specific antibody fragment linked to pseudomonas endotoxin A (26292(Fv)-PE38-KDEL) and an IL3:diphtheria toxin (IL-3:DT) fusion protein. CLL-1 has been targeted with naked antibodies resulting in Complement Dependent Cytotoxicity. CD33 has been targeted with several experimental therapeutics, including radionuclides, a scFvCD33:sTRAIL fusion protein and the clinically applied immunotoxin Gemtuzumab Ozogamicin (GO). After CD33-selective binding, GO internalizes and ends up in the lysosomes. In the acidic milieu of the lysosomes, the calicheamicin moiety is hydrolytically released. Next, calicheamicin translocates to the nucleus and intercalates with the DNA, resulting in apoptotic cell death. Therapeutic strategies targeting the death-receptors, TRAIL-R1/R2, include activating anti-TRAIL-R1 and anti-TRAIL-R2 antibodies, recombinant preparations of soluble TRAIL (sTRAIL) and scFv:sTRAIL fusion proteins. Activation of TRAIL-R1/-R2 receptors results in the recruitment of the adaptor molecule FADD and caspase-8 to the death domains of these receptors. Assembly of this so-called death-inducing signaling complex (DISC) leads to sequential activation of caspase-8 and caspase-3, ultimately resulting in DNA damage and apoptosis. In some cell types, caspase-8 can cleave Bid (tBid) and thereby activate a mitochondrial amplification loop. This loop is characterized by cytochrome C release from the mitochondria and subsequent the sequential activation of caspase-9 and caspase-3.*

Activation of apoptosis inducing receptors

The agonistic TRAIL death receptors, TRAIL-R1 and TRAIL-R2 (also known as DR4 and DR5, respectively) belong to the Tumor Necrosis Factor receptor family of death receptors. Ligation of these death receptors by TRAIL activates the extrinsic apoptotic pathway (reviewed in [43]). Hereupon, the adaptor protein FAS associated death domain (FADD) and the initiator caspase-8 are recruited to the intracellular death domains of these receptors. Assembly of this so-called death-inducing signaling complex (DISC)

leads to sequential activation of initiator and effector caspases and, ultimately, results in apoptotic cell death. In some cell types, the death-receptor pathway relies on an additional mitochondrial amplification loop that is activated by caspase-8-mediated cleavage of the BH3-only interacting domain death agonist (Bid) to a truncated form. Next, truncated BID (tBid) activates the mitochondrial pathway which involves cytochrome C and caspase-9. Intriguingly, apoptosis resulting from ligation of agonistic TRAIL-Rs appears to be restricted to malignant cells only, since normal cells appear to be resistant to TRAIL-R ligation. The mode of action of this tumor-selectivity of TRAIL-Rs ligation is still enigmatic.

Currently, activating anti-TRAIL-R1 and anti-TRAIL-R2 antibodies, as well as recombinant preparations of soluble TRAIL (sTRAIL) are evaluated in clinical trials [44-47]. In our laboratory, we have developed a series of fusion proteins, consisting of a tumor-selective scFv antibody fragment genetically fused to sTRAIL. These scFv:sTRAIL fusion proteins are designed to enhance tumor-selective cell surface delivery of sTRAIL (reviewed in [48]). Pre-clinically, these scFv:sTRAIL fusion proteins demonstrate potent and target antigen-restricted apoptosis induction. To this end, we have exploited both antigens expressed on solid tumors including Epidermal Growth Factor Receptor [49-51] and Epithelial Cell Adhesion Molecule [52;53], as well as antigens selectively expressed on leukemia cells including CD7 [54], CD19 [55] and CD33 [30].

Very recently, the expression of both TRAIL-R1 and TRAIL-R2 has been reported on LSCs. Comparing the gene expression profiles of AML-LSCs and HSCs, Majeti and colleagues demonstrated an increased expression of TRAIL-R1 and TRAIL-R2 in LSCs [56]. Furthermore, Yong et al. demonstrated that in CML non-cycling $CD34^+$ cells have a significant higher expression of TRAIL-R1 and TRAIL-R2 than cycling $CD34^+$ CML cells [57]. In line with this finding, sTRAIL significantly inhibited the clonogenic capacity of $CD34^+$ AML cells without affecting the clonogenic capacity of normal $CD34^+$ cells [58]. Together, these studies indicate that TRAIL-R1 and TRAIL-R2 receptors might be suitable candidates for the selective elimination of LSCs by targeted apoptosis induction. At present, we are assessing the efficacy of CD33- and CLL-1-selective scFv:sTRAIL fusion proteins for targeted apoptosis induction in AML-LSCs.

DISRUPTION OF THE LSCS-NICHE INTERACTION

In 1978, Schofield proposed that HSCs receive regulatory factors and signals within a specialized niche [59]. Currently, there are indications that these specialized niches are located in the bone marrow (BM), near the endosteum [60]. The endosteum is the inner surface located at the bone-BM interface and consisting mainly of osteoblasts and osteoclasts. More recent evidence indicates that also vascular and perivascular cells appear to contribute to these niche interactions as well (see review [61]). Signals within the HSCs niche regulate the normal survival, proliferation and differentiation of the HSCs. Recently, it was demonstrated that in leukemia the micromilieu in the HSCs niche appears to disturbed by the presence of Stem Cell Factor that is locally secreted by leukemia cells [62]. Furthermore, it has been demonstrated that intravenously injected AML-LSCs home to, engraft and subsequently reside in the endosteal region [63]. Such cellular tropism of AML-LSCs for a dedicate niche typically involves the coordinated signaling by various chemokines and adhesion molecules. Neutralization or inhibition of these signals may

be exploited for the selective elimination of LSCs. Below, a selection of recent studies is reviewed that collectively indicate that disruption of the LSCs-niche interactions may indeed form a therapeutic option for the elimination of LSCs (summarized in Fig. 3).

Targeting the Stroma-derived factor-1(SDF-1)/CXCR4 axis

Previously, it has been demonstrated that the chemokine SDF-1 (CXCL12) as expressed by reticular stromal cells in the BM is pivotal for homing of HSCs to their niche [64]. SDF-1 binds to the transmembrane receptor CXCR4 which is expressed on HSCs [64;65], leukemic blasts and AML-LSCs [66]. Of note, a high expression of CXCR4 in AML correlates with a negative prognosis [67;68]. The pivotal role of CXCR4 in retention of HSCs in the niche is clearly demonstrated by the mobilization of HSCs by the CXCR4 antagonist AMD3100 [69].

The interaction of leukemia cells with cell adhesion molecules in the niche typically reduces their sensitivity to cytotoxic therapies, a process known as cell adhesion-mediated drug resistance (CAM-DR). Inhibition of CXCR4 by the specific inhibitors AMD3100 and AMD3465 sensitized AML cells for cytotoxic therapies [70-72]. Most likely, treatment with the CXCR4 inhibitors mobilizes AML cells, thereby reducing CAM-DR and sensitizing the AML cells for cytotoxic therapy.

Tavor and colleagues demonstrated that the anti-CXCR4 mAb12G5 significantly inhibits homing of AML cells to the BM of recipient mice. Notably, treatment of mice with established AML with mAb12G5 reduced the leukemic burden in the BM, blood and spleen. Importantly, similar treatment did not affect the levels of engrafted HPCs [66].

Additionally, lowering available SDF-1 levels in leukemia might offer a therapeutic strategy. Recently, the medicinal herb-derived compound berberine was shown to inhibit SDF-1 production by stromal cells, thereby impairing the migration of AML blasts and AML-LSCs *in vitro* [73]. Together these results indicate the importance of a properly functioning SDF-1/CXCR4 axis for homing and retention of AML blasts and AML-LSCs. Therefore, the SDF-1/CXCR4 axis appears to be a suitable target candidate for the selective elimination of AML-LSCs.

Targeting Very Late Antigen 4 (VLA-4)

The β1-integrin, VLA-4, has a vital role in the retention of HPCs in the BM niche by immobilizing these cells [74]. The interaction of VLA-4 on AML cells with fibronectin on stromal cells initiates the phosphatidylinositol-3-OH kinase (PI3K) pro-survival pathway. Activation of the PI3K pathway in AML cells contributes to CAM-DR, which might explain the observation that VLA-4 expression on AML cells is correlated with a negative prognosis for AML patients. In line with this, Matsunaga and colleagues demonstrated that inhibition of VLA-4 interaction with fibronectin sensitized AML cells to cytosine arabinoside (Ara-C). This was demonstrated in a mice model for MRD in which they applied VLA-4 neutralizing antibodies to prevent the interaction with fibronectin [75]. Using the same MRD model, they recently demonstrated that a fibronectin-based synthetic peptide, designated FNIII14 [76], also sensitized AML cells to Ara-C [77]. Both approaches may be of considerable interest for disruption of the LSCs-niche interaction.

Targeting CD44

CD44 is a transmembrane receptor capable of binding several ligands, including hyaluronic acid which is abundantly expressed in the endosteal region [78]. On HSCs, CD44 serves as a homing receptor by interacting with E-selectin and L-selectin expressed in the BM [79]. Since CD44 is expressed on both CML-LSCs and AML-LSCs [80;81], neutralization of CD44-binding might impair the homing and retention of LSCs in the BM.

In CML, about 95% of the cases is characterized by the presence of the oncogenic fusion protein Bcr-Abl, a constitutively active mutant tyrosine kinase that causes uncontrolled cellular proliferation. The Bcr-Abl inhibitor Imatinib Mesylate induces remissions in the majority of CML patients. However, Imatinib appears to be unable to eradicate quiescent CML-LSCs [82], which might explain why the majority of CML patients relapse upon treatment discontinuation. Therefore, novel therapeutics for the selective elimination of Bcr-Abl$^+$ CML-LSCs are needed.

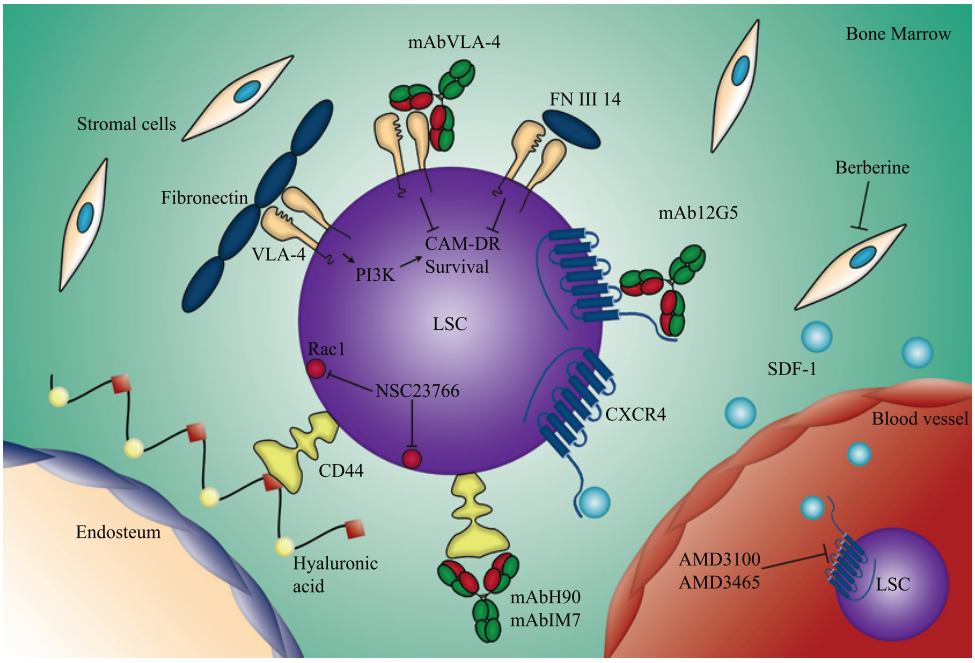

Figure 3. Disruption of the LSC-niche interaction. *The LSC-niche interaction can be disrupted by targeting VLA-4, CXCR4, CD44 and Rac1. Binding of LSCs-expressed VLA-4 to fibronectin in the BM initiates the PI3K pro-survival pathway and cell adhesion-mediated drug resistance (CAM-DR). The VLA-4-fibronectin interaction has been disrupted by mAbs and the synthetic peptide FNIII14. CXCR4 has been targeted with mAb12G5 and the specific inhibitors AMD3100 and AMD3465. Furthermore, the concentration of the CXCR4 ligand, SDF-1, can be lowered using berberine. The interaction of CD44 with the niche has been disrupted by mAbH90 and mAbIM7. The compound, NSC23766, has been utilized to specifically inhibit Rac1.*

In a series of elegant experiments, Krause and colleagues demonstrated that CD44 expressed on Bcr-Abl$^+$ CML-LSCs is essential for homing of these cells in recipient mice. Thereto, they performed a competitive homing assay in which the relative contribution of Bcr-Abl-transduced CD44$^+$ BM cells and Bcr-Abl-transduced CD44$^-$ BM cells in the development of CML was analyzed. Both cells types were mixed and injected intravenously

in irradiated recipients mice. After CML development, the animals were sacrificed and the relative contribution of the two distinct cell populations was analyzed. This revealed that Bcr-Abl-transduced $CD44^+$ BM cells contributed 5 times more to the development of CML than Bcr-Abl-transduced $CD44^-$ BM cells. CML development by the two different cell types was similar when injected intrafemorally. These results indicate that CD44 is necessary for homing of intravenously injected Bcr-Abl transduced cells to the BM. Importantly, anti-CD44 antibody treatment significantly prolonged survival of NOD/SCID recipient mice upon transplantation with CML-LSCs [80].
Simultaneously, the group of John Dick obtained similar results by targeting CD44 in AML. Treatment with the CD44-activating mAbH90 inhibited AML-LSCs homing to the BM niche and altered the fate of the AML-LSCs resulting in an efficient and selective elimination of the AML-LSCs [81]. Together, these studies clearly indicate a rationale for targeting CD44 to selectively eliminate CML-LSCs and AML-LSCs. Therefore, an evaluation of the efficacy of targeting CD44 in both CML and AML patients seems warranted.

Targeting Rac
The Rac subfamily of the Rho-family of GTP-ases is implicated in cell adhesion and motility. The family consists of three members Rac1, Rac2 and Rac3. Rac1 is ubiquitously expressed [83], while Rac2 expression is limited to the hematopoietic system [84] and Rac3 is selectively expressed in the brain [85]. $Rac1^{-/-}$ HSCs and progenitors are unable to reconstitute hematopoiesis in irradiated recipient mice upon transplantation [86]. Moreover, it has been shown that Rac is overexpressed on $CD34^+$ cells derived from AML patients. Rac appears to contribute to an enhanced migration and adhesion capacity of $CD34^+$ cells to BM stromal cells.
Gao and colleagues [87] generated a Rac1 inhibitor, designated NSC23766, that significantly delayed leukemia development in a murine tumor model [88] and strongly reduced the clonal expansion of $CD34^+$ AML cells [89]. Together, these promising data warrants further pre-clinical assessment of Rac1 inhibition for the elimination of LSCs.

TARGETING ABERRANT MOLECULAR PATHWAYS

Both, HSCs and LSCs rely on similar molecular pathways for their maintenance and self-renewal. However, in LSCs these pathways are often aberrantly regulated. LSCs appear to be particular dependent on these deregulated pathways to maintain their malignant phenotype. This strong dependence indicates that therapeutic intervention in these pathways might be exploitable for the selective elimination of LSCs. Thus far, several of these deregulated pathways have been identified (e.g. the Hedgehog and Wnt pathways). The selective targeting of these pathways may yield promising anti-LSCs activity.
Compounds such as arsenic trioxide and parthenolide have been assessed for their capacity to induce apoptosis in LSCs. However, the pathways in which these compounds intervene are currently not completely understood. Therefore, further delineation of their specific molecular targets may contribute to development of more and rationally designed LSCs targeted therapeutics. Below, we provide a selection of aberrantly regulated pathways present in LSCs and a selection of chemical compounds that may have potential for the elimination of LSCs (summarized in Fig. 4).

The Hedgehog (Hh) pathway

The Hh pathway is involved in HSCs self-renewal [90] and is initiated by binding of one of the three Hh ligands, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) or desert Hedgehog (Dhh) to the transmembrane receptor Patched. Upon binding of Hh to Patched the inhibiting effect of Patched on the transmembrane receptor Smoothened (Smo) is relieved (reviewed in [91]). Next, Smo can activate Hh targets genes via the Gli family of transcription factors. Amongst these Hh targets genes are CyclinD/E [92] and B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) [93] which are involved in cell proliferation and self-renewal, respectively. The Hh pathway can be inhibited by the chemical compound cyclopamine, which stabilizes Smo in its inactive form [94]. In a very recent study conducted by Zhao and colleagues the role of Smo in Bcr-Abl^{+} CML-LSCs was analyzed [95]. Thereto, Bcr-Abl-transduced normal HPCs and Bcr-Abl-transduced Smo$^{-/-}$ HPCs were transplanted in irradiated recipient mice after which the development of CML was compared. More than 90% of the mice transplanted with Bcr-Abl-transduced normal HPCs developed CML. In contrast, only 47% of the mice developed CML when transplanted with Bcr-Abl-transduced Smo$^{-/-}$ HPCs. When the Bcr-Abl-transduced HSCs were transplanted in mice, all recipient mice died from CML within four weeks. However, when these transplanted mice were treated with cyclopamine, 60% of the animals were still alive after 7 weeks. Furthermore, CML cells freshly isolated from cyclopamine-treated mice were unable to effectively repopulate secondary recipients. This corroborates with the strong reduction (up to 14-fold) of CML-LSCs in cyclopamine-treated mice. Additionally, cyclopamine treatment of human CML cell lines and primary CML samples reduced their clonogenic capacity. Importantly, the authors indicate that Smo inhibition by cyclopamine may also impaired the propagation of Imatinib-resistant human CML cells in xenografts [95].

The Wnt pathway

Wnt is implicated in HSCs self-renewal and proliferation [96] and is overexpressed in many different types of human cancers [97]. Thus far, three distinct Wnt signaling pathways have been described of which the canonical pathway is most prominently involved in leukemia. The key mediator of the canonical pathway is β-catenin. Normally, β-catenin is degraded rapidly after synthesis by the multiprotein destruction complex (MDC), which tags β-catenin for proteasomal degradation. The MDC consists of axin, adenomatous polyposis coli (APC) and glycogen-synthase kinase 3β (GSK-3β). Upon binding of Wnt to its receptors Frizzled and LRP5/6, the MDC becomes destabilized and therefore unable to tag β-catenin for degradation. Consequently, β-catenin accumulates and is able to translocate to the nucleus. In the nucleus β-catenin complexes with T-cell factor (TCF), B cell lymphoma 9 (Bcl-9) and pygo to induce TCF target genes expression [98], including the oncogenes C-MYC [99], Cyclin-D1 [100] and the cell surface antigen CD44 [101].

In AML, several oncogenic fusion proteins, such as AML-1/eight twenty one (AML1-ETO), promyelocytic leukemia / retinoic acid receptor alpha (PML/RARα) and promyelocytic leukemia zinc finger (PLZF/RARα) [102] can activate the Wnt signaling pathway. Furthermore, evidence is emerging that indicate an aberrant Wnt signaling pathway in LSCs [56] (extensively reviewed in ref [103]). In this respect, it has been shown that β-catenin plays a pivotal role in self-renewal of both HSCs as well as CML-LSCs [104]. Furthermore, very recently β-catenin has been implicated in the survival of Imatinib

resistant CML-LSCs in Bcr-Abl induced CML [105]. Therefore, modulation of Wnt signaling in the LSCs may contribute to their selective elimination.
There are several possibilities to inhibit the Wnt signaling pathway. Wnt signaling can be inhibited by extracellular proteins (reviewed in [106]) which scavenge Wnt and thereby prevent Wnt from binding to its receptors. These Wnt binding proteins include secreted Frizzled-related proteins (sFRP), Wnt inhibitory factor 1 (wif-1) and Cerberus. Moreover, members of the Dickkopf (DKK) family can directly bind the Wnt receptor and thereby prevent Wnt-induced signaling. In this respect, very recently it appeared that the anti-proliferative effects of human mesenchymal stem cells towards CML cells is at least partly attributable to secretion of DKK-1 [107]. Additionally, also very recently two natural compounds, CGP049090 and PKF 115-584 [108], have been identified that disrupt the TCF/β-catenin complex. In AML these compounds possess potent tumoricidal activity [109]. It would be worthwhile to assess the possibilities of inhibition of the Wnt signaling pathway for the elimination of LSCs.

Aldehyde dehydrogenases (ALDH) enzymes
ALDH are a group of cytosolic enzymes that catalyzes the conversion of intracellular aldehydes. The ALDH superfamily consists of seventeen members [110] of which two members, cytosolic aldehyde dehydrogenase class-1A1 (ALDH1A1) and class-3A1 (ALDH3A1), have been implicated in tumor drug resistance against oxazaphosphorines (e.g. cyclophosphamide) [111]. ALDH enzyme activity, including the activity of ALDH1A1 and ALDH3A1, can be assessed using activatable fluorescent substrates. ALDH enzyme activity measurements enables the identification and isolation of HSCs [112], breast cancer stem cells [113], liver cancer stem cells [114] and AML-LSCs [115]. Manipulation of ALDH1A1 and ALDH3A1 expression or activity may be used to sensitize AML-LSCs to chemotherapy, especially to oxazaphosphorines. In this respect, the ALDH1 inhibitor, diethylamino-benzaldehyde (DEAB) might be utilized for sensitizing AML-LSCs to treatment with chemotherapeutic agents. Unfortunately, DEAB appeared to be very instable *in vivo* [116] . Therefore, the development of novel DEAB-analogues with enhanced stability is warranted. All-trans Retinoic Acid (ATRA), an established clinically applied anti-leukemia agent, appears to downregulate both ALDH1A1 and ALDH3A1 which may be beneficial for the selective elimination of LSCs [111]. In this respect, treatment of Acute Promyelocytic Leukemia (APL) and AML cells with ATRA resulted in differentiation of tumor cells and stimulation of cell cycling [117]. Therefore, we suggest that the combination treatment of oxazaphosphorines and ATRA may have potential for the elimination of LSCs.

Phosphatase and tensin homologue (PTEN)
PTEN is the second most commonly mutated gene in human malignancies, including hematopoietic malignancies [118;119]. Normally, PTEN inhibits cell proliferation and cell survival by negatively regulating the PI3K pathway [120]. In this respect, Yilmaz and colleagues recently compared the effect of PTEN inactivation in HSCs and LSCs [121]. Conditional deletion of PTEN resulted in the development of myeloproliferative disorder in 17 out of 19 mice which in the majority culminated in leukemia. In the HSCs, deletion of PTEN was characterized by an elevated cell cycle progression and an impaired replenishment of the HSCs pool, thus finally resulting in a depletion of HSCs. Apparently, PTEN deletion facilitates leukemogenesis while it depletes HSCs. These effects of PTEN

deletion appear to be mediated by mammalian target of rapamycin (mTOR), since treatment with the mTOR inhibitor rapamycin reversed the effects. Therefore, rapamycin treatment seems to be a promising approach for the selective elimination of LSCs while sparing HSCs.
However, Ito and colleagues recently reported on a contradictory effect of rapamycin [122]. They investigated the role of the tumor-suppressor promyelocytic leukemia protein (PML) in CML. Their results demonstrated that PML-deficient CML-LSCs are less quiescent than wild-type CML-LSCs, resulting in LSCs exhaustion. Furthermore, deletion of PML in HSCs also results in exhaustion and consequently impairment in long-term hematopoietic reconstitution. Since PML deficiency in both HSCs and LSCs is characterized by an increased mTOR activity they assessed the effect of rapamycin treatment. Here, rapamycin rescued the typical PML-deficient phenotype, thus exhaustion of the CML-LSCs and HSCs was prevented. Together, these seemingly contradictory results of mTOR inhibition in the elimination of LSCs indicate that further delineation of the role of mTOR in LSCs is needed.
Of note, since arsenic trioxide is known to decrease PML levels, Ito and colleagues continued with assessing arsenic trioxide for the elimination of CML-LSCs [122]. Treatment with arsenic trioxide resulted in the exit of the CML-LSCs from quiescence and therefore the CML-LSCs might become exhausted and more vulnerable to chemotherapeutics. Importantly, this cell cycle entry appeared to be more profound in LSCs than in HSCs, thereby generating a possible therapeutic window for the treatment of CML-LSCs by arsenic trioxide.

4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione (TDZD-8)
TDZD-8 was designed as a non-ATP competitive inhibitor of GSK-3β for the treatment of Alzheimer's disease [123]. However, Guzman and colleagues uncovered potent tumoricidal activity of TDZD-8 towards leukemia cells. In a series of experiments they demonstrated that TDZD-8 exhibit strong anti-leukemia effects towards AML, CLL, ALL and blast crisis CML cells, without apparent toxic effects towards normal BM cells. Since a panel of other GSK-3β inhibitors did not possess any comparable activity, the anti-leukemia effect is probably not governed by the inhibition of GSK-3β. This notion is supported by the observation that β-catenin, of which the degradation is in part mediated by GSK-3β, is elevated in CML-LSCs. Moreover, very recently GSK-3β missplicing was observed in CML-LSCs and not in HSCs [124], together indicating that GSK-3β is dispensable for leukemogenesis.
Importantly, TDZD-8 induced apoptosis in AML-LSCs and CML-LSCs without affecting HSCs [125]. In line with this, TDZD-8 specifically reduced the engraftment potential of AML-LSCs and not of HSCs. TDZD-8 is a highly hydrophobic agent which may result in a rapid loss of membrane integrity in leukemic cells by TDZD-8 insertions in the cellular membrane. The absence of this effect in HSCs might indicate that there is crucial difference in the membrane composition of LSCs and HSCs [125], which might open novel avenues for the selective elimination of LSCs.

Parthenolide (PTL)
PTL is a compound, derived from the plant feverfew, which eliminates cancer cells via reactive oxygen species and the inhibition of NF-kB [126-128]. Guzman and colleagues

analyzed the effects of PTL and Ara-C on AML blasts and AML-LSCs. Apoptosis induction in the AML blasts by Ara-C was more pronounced than by PTL. However, PTL potently induced apoptosis in AML-LSCs, whereas Ara-C exhibited only minimal apoptotic activity. Importantly, the PTL concentration necessary for significant AML-LSCs elimination had only a modest effect on HSCs [129]. Moreover, PTL treatment significantly reduced the engraftment potential of AML-LSCs, while PTL treatment did not affected the engraftment of HSCs [129].

Despite the favorable characteristics, the clinical application of PTL is probably hampered by its poor water solubility. Therefore, Guzman and colleagues generated a series of parthenolide analogues aiming to identify an active compound with improved pharmacological properties [130]. Recently, they identified a novel compound, designated dimethylamino analog of parthenolide (DMAPT), which exhibits similar activity as PTL. However, when formulated as a fumarate salt, DMAPT had 1000-fold enhanced water solubility, resulting in a 70% bioavailability of orally applied DMAPT [130]. In contrast to PTL, oral administration of DMAPT did result in plasma concentrations sufficient to exert an anti-leukemia effect in both rodent and canine models.

EPIGENETIC REPROGRAMMING OF LSCS

The step-wise conversion of a normal cell to a leukemic cell typically involves the activation of oncogenes and anti-apoptotic genes and the inactivation of tumor suppressor and pro-apoptotic genes [131]. Frequently, gene expression in leukemic cells is altered by epigenetic reprogramming (reviewed in [132]).

Epigenetic reprogramming is characterized by various distinct chromatin modifications. Chromatin consists of histone proteins and DNA. The central unit of the chromatin structure is an octamer of core histone proteins, referred to as the nucleosome. The distance between adjacent nucleosomes and thus the compaction of the chromatin in between, defines two types of chromatin. Euchromatin is the open type of chromatin with a relative low compaction which allows access of the transcription machinery to the DNA, while heterochromatin is the condensed form in which the transcription machinery can not access the DNA and reflects an inactive state. There are several types of modifications that can alter the accessibility of the transcription machinery to the DNA. The best studied are DNA and histone methylation which are associated with heterochromatin and histone acetylation which is associated with euchromatin.

Normal stems cells rely on Polycomb group (PcG) proteins to repress genes encoding transcription factors required for differentiation including the homeotic (HOX) genes [133]. It is postulated that methylation of promoter regions of those repressed genes could lock in stem cell phenotypes and initiate abnormal clonal expansion and thereby predispose to cancer [134;135]. Repression of genes by the PcG proteins is initiated by the Polycomb Repressor Complex 2 (PRC2) containing the histone methyltransferase EZH2, after which the Polycomb Repressor Complex 1 (PRC1) further maintains the repression. The PRC1 comprises amongst others Bmi-1, which is essential for self-renewal of HSCs [136]. Of note, in both AML and CML patients Bmi-1 is overexpressed compared to normal CD34+ BM cells. Patients with an elevated Bmi-1 expression have less chance of achieving complete remission, higher chance of relapse and reduced duration of survival [137;138]. Moreover, very recently Wang and colleagues have shown that the dysregulation of a

chromatin-binding plant homeodomain (PHD) finger can cause hematological malignancies [139]. Normally, PHD fingers respond to certain histone methylation states [140;141]. In AML however, chromosomal translocations are observed which results in the fusion of a PHD finger to a common leukemia fusion partner called NUP98 [142-144]. Wang and colleagues compared the transcriptome profile of BM progenitor cells transduced with such a fusion protein with control cells. This analysis indicated that in the cells transduced with this fusion protein several genes were upregulated including HOXA9 and MEIS1 [145]. Lessard and Sauvageau have shown that enforced expression of HOXA9/MEIS1

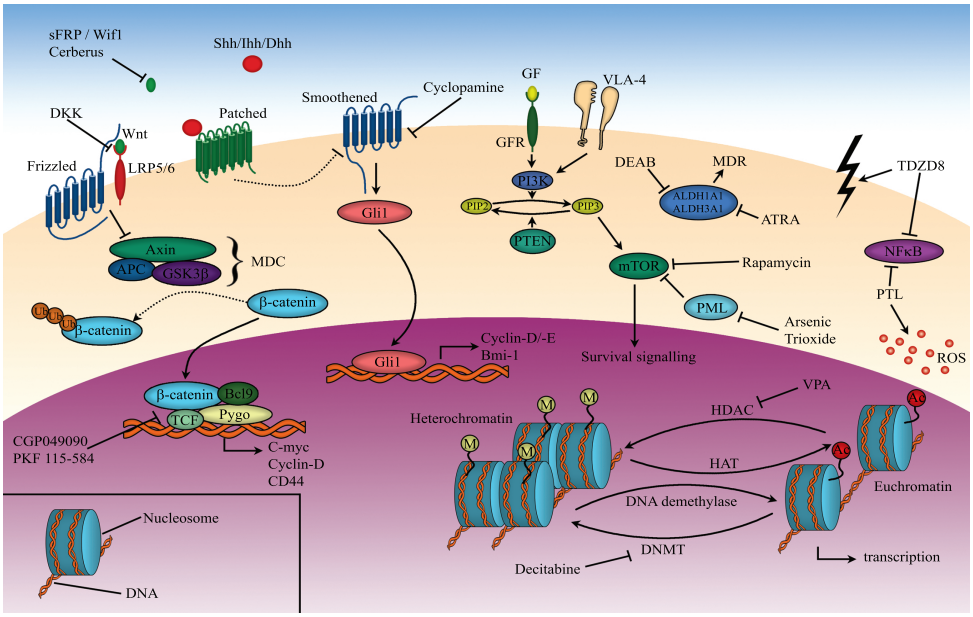

Figure 4. Targeting aberrantly regulated pathways and epigenetic reprogramming *Aberrantly regulated Wnt and Hedgehog pathways have been identified in LSCs and therefore might be suitable candidates for therapeutic intervention. At the cell surface, binding of Wnt to LRP5/6 and Frizzled destabilizes the Multiprotein Destruction Complex (MDC), which in the absence of Wnt signaling facilitates the degradation of β-catenin. As a result of Wnt binding to its receptors, β-catenin accumulates and translocates to the nucleus where it complexes with TCF, Bcl-9 and pygo to induce the expression of several genes including c-myc, cyclin-D and CD44. Extracellularly, the Wnt signaling pathway can be inhibited by the scavengers sFRP, Wif1 and Cerberus and the DKK-family members which prevent Wnt binding to its receptor. Intracellularly, Wnt signaling can be inhibited by the compounds CGP049090 and PKF115-584, which disrupt the TCF/β-catenin complex. The Hedgehog pathway is initiated by binding of a ligand (Shh, Ihh or Dhh) to the receptor Patched. Hereupon, Smoothened becomes activated and via the Gli family of transcription factors induces the expression of several target genes including CyclinD/E and Bmi-1. The Hedgehog pathway can be inhibited by cyclopamine which stabilizes Smoothened in its inactive form. Binding of Growth factors (GF) to Growth Factor Receptors (GFR) or binding of VLA-4 to fibronectin induces activation of PI3K. This pro-survival signaling pathway is characterized by the conversion of PIP2 to PIP3 and subsequent downstream signaling via several proteins, including mTOR. Normally, PTEN functions as an inhibitor of the PI3K pathway. Pharmacologically, both rapamycin and arsenic trioxide (via PML) can alter mTOR activation. The enzymes ALDH1A1 and ALDH3A1 have been implicated in Multi Drug Resistance (MDR) and can be inhibited by DEAB and ATRA. The anti-leukemia activity of parthenolide (PTL) is characterized by the inhibition of NF-κB and the generation of ROS. The compound TDZD-8 can also inhibit NF-κB activation and might influence LSCs membrane integrity. Frequently, gene expression in leukemic cells is altered by epigenetic reprogramming. Therefore, reprogramming the epigenetic status using decitabine or HDACi appears promising for the elimination of LSCs.*

can induce leukemia, which is dependent on the expression of Bmi-1 [146], suggesting that Bmi-1 inhibitors may contribute to the elimination of LSCs.

However, also drugs with a more general epigenetic modification activity may have clinical relevance in attacking LSCs. This is evident from the clinical application of the demethylating agent decitabine, which is approved for the treatment of MDS. Decitabine inhibits DNA methyltransferases (DNMT) and therefore has a broad range of action by tipping the balance towards euchromatin. Intriguingly, decitabine exerts differential effects in LSCs and HSCs, which is probably due to differences in their epigenomic status. In LSCs treatment with decitabine results in differentiation, whereas decitabine treatment of HSCs results in enhanced self-renewal (Negrotto S et al. ASH Annual Meeting Abstracts 2008;112:201). Furthermore, it was demonstrated that decitabine treatment reactivated more genes in bladder cancer cells than in normal fibroblasts [147]. These studies indicate that general epigenetic modifications can exert differential effects in LSCs and HSCs, which might be utilized for the selective elimination of LSCs.

Other general epigenetic modulators are HDACi such as suberoylanilide hydroxamic acid (SAHA), 4-phenylbutyric acid (PBA) and VPA. HDACi can also tip the balance toward euchromatin and have shown to induce apoptosis in leukemia (see Fig. 4). In clinical trials VPA monotherapy was well tolerated, but showed disappointing anti-leukemia effects (reviewed in [148]). However, combination treatment of VPA with other anti-cancer agents showed significantly enhanced and sometimes synergistic anti-tumor effects [35;149-151].

MicroRNA's (miRs) are small, non-coding, single stranded RNA sequences that regulate gene expression by binding to mRNA molecules. Interestingly, upon co-treatment with decitabine and PBA, miRs appear to be differential reactivated in cancer cells and normal cells [152]. Saito and colleagues have shown that the transcription of one particular miR, miR-127, was increased upon combination treatment of decitabine and PBA. Subsequently, they identified that a predicted target of miR-127, BCL6, was downregulated [152]. This indicates that not only epigenetic silenced protein-coding sequences, but also miRs can be reactivated upon treatment with demethylating agents and HDACi. This opens novel avenues for modifying proteins levels in LSCs and therefore may be exploited to eliminate LSCs.

CONCLUSIONS AND PERSPECTIVES

The specific elimination of LSCs in hemato-oncology is a research area still in its infancy. Currently, researchers are at the brink of identifying biological processes imperative to LSCs biology. Elucidation of these LSCs-specific characteristics is of eminent importance for the rational design of novel LSCs targeted therapies. It is anticipated that the successful elimination of LSCs will have tremendous impact on leukemia therapy. Several strategies appear promising for the elimination of LSCs, including targeting LSC-selective cell surface antigens, disrupting LSCs-niche interactions, targeting aberrantly regulated pathways and epigenetic reprogramming of LSCs. Several studies utilizing these strategies have generated promising pre-clinical results. However, the vast majority of the data is obtained by xenografting LSCs in severely immunocompromised recipient mice. It is evident that the data obtained using these models can not be extrapolated directly to the clinical situation. Furthermore, it is likely that multifaceted therapy combining several

strategies will be the most effective approach. Therefore, rational designed combinatorial approaches and multi-modality targeting of LSCs are warranted. Subsequent, these combinatorial strategies have to prove to be safe and effective in clinical trials.

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